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(54) Title: MULTIPLEXED LIGAND/PROTEIN BINDING ASSAYS WITH PNA LABELS

(57) Abstract: Multiplexed methods for discovering and quantifying ligands, proteins and ligand-protein interactions are disclosed. In one embodiment, one or more ligand candidates, preferably from a combinatorial library and each labeled with a unique peptide nucleic acid (PNA), are combined simultaneously with one or more proteins, and complementary ligand/protein combinations are discovered. The methods of the invention provide the advantages of ultra-multiplicity, such as testing an entire combinatorial synthesis library all at once against an entire proteome; practicality, since unlabeled proteins can be tested; and ultra-sensitivity, in that a proteome from a single cell can be studied. The methods of the invention make possible advances in protein chips and multiplexing competitive and noncompetitive ligand assays that use known proteins as binding reagents. They also enable ligand-based sandwich assays of protein analytes.

TITLE OF THE INVENTION

MULTIPLEXED LIGAND/PROTEIN BINDING ASSAYS WITH PNA LABELS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Patent Application No. 60/282,868 filed April 10, 2001, entitled, COMBINATORIAL PROTEOMICS, the whole of which is hereby incorporated by reference herein.

FIELD OF THE INVENTION

The field of the invention is detection of binding between proteins and ligands for the purpose of ligand or protein discovery and/or quantitation.

BACKGROUND OF THE INVENTION

Investigations in the field of proteomics seek to discover, monitor and understand proteins, individually and globally, including their mutual interactions and interactions with other biomolecules and biomolecule analogs. Proteins function largely through these interactions, and their small molecule-binding partners are generally termed complementary molecules or ligands. Small-molecule drugs, for example, are an important class of ligands, and interaction with their protein binding partners is usually the basis for their function. It is well recognized that there is a great need for improved techniques in proteomics (Borman, S., Any New Proteomics Techniques Out There? Chem. Eng. News, Nov. 26, 2001, 27-29).

Many drugs have been discovered by screening natural products as potential protein ligands, but, increasingly, combinatorial libraries of synthetic compounds are being created and tested for this purpose (Borman, S., Combinatorial Chemistry, Chem. Eng. News, Aug. 27, 2001, 49-58). In order to discover

lead compounds from a combinatorial library, usually a target protein of therapeutic interest is labeled with a signal group such as a fluorescent dye, enzyme or radioisotope and then an assay is set up to see which compounds in the library are ligands for the protein. One way of making this examination more efficient is to print the library numbers onto a solid surface in a miniaturized way to speed up and lower the cost of the screening process (MacBeath, G., Koehler, A. N., Schreiber, S. L., Printing Small Molecules as Microarrays and Detecting Protein-Ligand Interactions en Masse, J. Am. Chem. Soc., 121, 7967-7968 1999). However, this requires the purification and signal labeling of each protein of interest. To some degree, the use of different signal groups can enable multiple proteins to be tested simultaneously, but the similarity of conventional signal groups has greatly limited the multiplexing (multiplicity) of this approach.

Mass spectrometry (MS) has emerged as a useful technique in recent years for studying proteins, for example in combination with other techniques such as electrophoresis or high performance liquid chromatography. However, the sensitivity and speed of MS is limited, and it needs to be combined with other methods for discovery of complementary ligand/protein pairs. Protein chips are also being developed as measurement tools for proteomes, but these chips have been limited by problems such as protein denaturation, nonspecific binding, and the need to label the target proteins (Service, R. F., Searching for Recipes for Protein Chips, Science, 294, 2080-2082, 2001). Corresponding chips for DNA analysis, in which spots of DNA are arrayed on a solid surface to bind complementary DNA sequences, have been much more successful (Zubritsky, E., Spotting a microarray system, Anal. Chem. A, 761-767A, 2000).

Beyond the discovery phase of ligand/protein binding partners, there is a need to quantify both proteins and ligands. The above techniques can be used for this purpose as well, but the

same limitations persist. Conventional ligand assays such as radioimmunoassay and enzyme linked immunosorbent assay (ELISA) can be used to quantify ligands and proteins. However, these assays are limited in their ability to measure multiple ligands or proteins simultaneously. While some solid phase versions of these assays can boost analyte multiplicity, nonspecific binding interferences then tend to worsen along with assay speed and cost. The long-sought goal of developing a practical, solution-based, highly-multiplexed ligand assay technique for quantifying a multiplicity of ligands or proteins simultaneously in the same sample has yet to be reached.

BRIEF SUMMARY OF THE INVENTION

The invention is directed to multiplexed methods for discovering and quantifying ligands, proteins and ligand-protein interactions and to novel peptide nucleic acid-tagged ligands for carrying out such methods. The methods of the invention provide the advantages of ultra-multiplicity, such as testing an entire combinatorial synthesis library all at once against an entire proteome; practicality, since unlabeled proteins can be tested; and ultra-sensitivity, in that a proteome from a single cell can be studied. The methods of the invention make possible advances in protein chips and in multiplexing competitive and noncompetitive ligand assays that use known proteins as binding reagents. They also enable ligand-based sandwich assays of protein analytes.

In one embodiment, one or more ligand candidates, preferably from a combinatorial library and each labeled with a unique peptide nucleic acid (PNA), are combined simultaneously with one or more proteins, and complementary ligand/protein combinations are discovered. PNAs are advantageous as labeling entities because of their small size, high coding properties, ease of detection by means of hybridization and opportunity for ultrasensitivity based on detection by a polymerase chain reaction (PCR) or related nucleic acid amplification reaction.

For example, complementary (positive) PNA-ligand/protein complexes can be separated by ultrafiltration from nonbonding (negative) PNA-labeled ligands, followed by dissociation of the positive complexes, and then hybridization-based detection of the released positive PNA-ligand species on an array of PNA or DNA spots on a solid surface can be carried out.

In a second embodiment, one or more PNA molecules are used as labels in a multiplexed competitive or noncompetitive ligand assay in which known binding proteins are employed to quantify known analytes. A second label such as biotin, a common PNA sequence, or a fluorescent tag is employed as well. As above, detection takes place via hybridization of the PNA labels onto an array of complimentary PNA or DNA spots on a solid surface.

The methods of the invention bring together, for the first time in the field of proteomics, the techniques of combinatorial synthesis, peptide nucleic acid coding, detection signaling, ultrafiltration separation, PNA (or DNA) detection assays, multiplexed ligand assays and detection of nonlabeled proteins. Furthermore, the invention gives rise to technology that can be used for simultaneous multiplexed ligand/multiplexed protein discovery and also ligand and protein quantitation.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims, taken in conjunction with the accompanying drawings, in which:

Fig. 1 depicts a synthetic scheme for the preparation of ligand-PNA-signal (LPS) species;

Fig. 2A depicts a scheme for ligand/protein discovery or protein quantitation. After LPS_2 is removed by ultrafiltration, protein/LPS_1 is dissociated, and LPS_1 is detected via a hybridization reaction;

Fig. 2B depicts a scheme for detecting positive LPS species by PCR (continuation of step 3b in Figure 2A); and

Fig. 3 depicts three kinds of ligand assays (I, II, III) for measuring analyte (A) in which the binding protein is an antibody (Ab) and the coding substance for multiplicity is a peptide nucleic acid (PNA). Also shown (IV) are two versions of a sandwich ligand assay for measuring protein analytes. (S = signal group, L = ligand.)

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the invention, complementary protein/ligand binding pairs are discovered. A sample containing one or more proteins is combined, in solution or suspension, with one or more ligand-PNA-signal (LPS) species according to the invention. Each LPS species comprises a ligand part, L, covalently attached to a peptide nucleic acid (PNA) and a signal group (S), such as a biotin or biotin-analog (B). Each PS tag is individually identifiable separately from every other PS tag by the identity of the incorporated PNA species. Protein/LPS complexes then form between complementary proteins and ligands, which can be referred to as "positive" proteins and "positive" LPS species for each pair. These complexes are then separated from non-complexed LPS species, those that have failed to find a complementary protein partner, and dissociated. Decoding of each positive LPS species enables the identity of its ligand part to be revealed.

Peptide nucleic acids (PNAs) are DNA mimics in which the backbone, which is substituted with DNA nucleobases, is composed of N-(2-aminoethyl)glycine rather than sugar-phosphate units. PNAs hybridize to complementary DNA and RNA molecules, and to each other. Usually, the hybridization products are stronger with PNAs. Since PNAs can be synthesized on the same instruments that are used for automated synthesis of peptides, and PNA synthons (monomers for the synthesis) for this purpose are available commercially, PNAs are readily available. Compounds have been

synthesized in this way which are part peptide and part PNA (Bau, S. and Wickstrom, E., Synthesis and Characterization of a Peptide Nucleic Acid Conjugated to a D-Peptide Analog of Insulin-like Growth Factor 1 for Increased Cellular Uptake, Bioconjugate Chem., 8, 481-488, (1997). An automated, parallel synthesis system has been set up which prepares 1536 different PNAs per run (Matysiak, S., Reuthner, F., Hoeisel, J. D., Searching for Recipes for Protein Chips, BioTechniques, 31, 896-904, 2001). PNAs have been substituted with other functional groups such as biotin and maleimide. A formula is available for predicting thermal stability of PNA/DNA duplexes (Giesen, U., Kleider, W., Berding, C., Geiger, A., Orum, H., Nielsen, P. E., A formula for thermal stability (Tm) prediction of PNA/DNA duplexes, Nucleic Acids Res., 26, 5004-5006 1998, which will be useful to select sequences and lengths of PNAs that work well together in multiplexed PNA assays of the invention. Usually PNAs are prepared in a length of about 5 to 25 bases. PNAs are readily detected by mass spectrometry. Various applications of PNAs have been described (Neilsen, P. E., Applications of peptide nucleic acids, Current Opinion in Biotechnology, 10, 71-75, 1999) and PNA arrays have been prepared for hybridization-based screening of DNA (Weiler, J., Gausepohl, H., Hauser, N., Jensen, O. N., Hoheisel, H. D., Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays, Nucleic Acids Research, 25, 2792-2799, 1997).

Wide variation in functional group attachment to PNAs is possible. For example, the length, polarity, type and location of functional groups on a PNA molecule, and also the type and location of structural branch points can readily be selected. To make the PNA or a functional group of an LPS species more accessible sterically, glycine spacers can be introduced at positions in the LPS adjacent to the PNA or functional group. In turn, to make an LPS species more polar, serine rather than glycine spacers can be used. Lysine, aspartic acid, glutamic acid and related residues can be employed to establish branch points.

Because many reactions are available from the bioconjugation literature to convert (usually by synthetic extension) one functional group into another (e.g., reaction of a primary amine with succinic anhydride converts, by extension, the amino functional group into a carboxyl functional group), different moieties such as ligands and signal groups can be set up in various configurations on PNAs to optimize the chemical and physical properties of the LPS species for a given application. Often PNAs and PNA derivatives are made by solid-phase synthesis on a resin bead, and are capped with protecting groups, except for the primary amino group at the unattached end, just before release. At this stage in the synthesis (just before release), it is convenient to convert this primary amino group into another functional group if desired.

An exemplary LPS species for use in methods of the invention can be prepared as shown in Fig. 1. The starting compound, W-Lys-X **10**, is prepared by a procedure similar to that described in Basu, S., and Wickstrom, E., Synthesis and Characterization of a Peptide Nucleic Acid Conjugated to a D-Peptide Analog of Insulin-like Growth Factor 1 for Increased Cellular Uptake, Bioconjugate Chem., 8, 481-488, 1997, except that: (1) a biotinylated lysine is incorporated during the synthesis; (2) the numbers of glycine and other amino acid spacers can be varied (e.g., l, m, and n $\geq$ 0); and (3) the resin attached precursor, which terminates in a free amino group, is reacted before release from the resin with succinic anhydride to form W-Lys-X. The biotinylated lysine synthon can be prepared by reacting N_α-Fmoc-N_ε-Boc-lysine (Sigma) with biotinamidohexanoic acid N-hydroxysuccinimide ester (Sigma), and then converting the product, after acidic removal of the Boc group, to an active ester via an activator such as O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

W-Lys-X is then reacted with N-hydroxysuccinimide-Y-maleimide **12**, which can be prepared by reacting ε-maleimidocaproic

acid NHS ester (Sigma, St. Louis, MO) with ε-aminocaproic acid followed by reaction with N-hydroxysuccinimide in the presence of N, N-dicylohexylcarbodiimide, to form Z-maleimide **14**, where Z is the combination of W-Lys-X with Y. Reaction of each Z-maleimide compound (having a unique PNA sequence) with a unique ligand that bears a sulfhydryl (thiol) group yields a series of LPS species **16**. Such thiol-ligands can be obtained from combinatorial synthesis products as described (MacBeath, G., Koehler, A. N., Schreiber, S. L., Printing Small Molecules as Microarrays and Detecting Protein-Ligand Interactions en Masse, J. Am. Chem. Soc., 121, 7967-7968, 1999).

Many variations of the scheme shown in Fig. 1 can be practiced to prepare other LPS species. For example, the lysine side chain of W-Lys-X can be capped with acetic anhydride, forming W-N-acetyl-Lys-X, and the carboxyl group of W then can be coupled to ethylenediamine followed by reaction with ε-maleimidocaproic acid NHS ester. The LPS products in this case could be substituted at opposite ends with biotin and a thiol ligand. If combinatorial ligands are available with amino functional groups, the carboxyl end of W-N-acetyl-Lys-X can be activated with a carbodiimide for coupling to such ligands. Alternatively, the carboxyl end of W-Lys-X can be capped by similar activation in the presence of excess ethanolamine, and then the uncapped lysine can be reacted with succinic anhydride followed by carbodiimide activation of the resulting new carboxyl moiety for reaction with ligands containing a primary amine functional group. Reaction of the lysine side chain of W-Lys-X with S-acetylthioacetate followed by hydroxylamine would provide a thiol group at this site for reaction with combinatorial ligands bearing a maleimide functional group. Combinatorial ligands with carboxyl functional groups (e.g., Tan, D. S., Foley, M. A., Shair, M. D., Schreiber, S. L., Steroselective Synthesis of over Two Million Compounds Having Structural Features Both Reminiscent of Natural Products and Compatible with Miniaturized Cell-based Assays, J. Am. Chem. Soc.,

120, 8565-8566, 1998) can be converted to active esters for coupling onto W-Lys-X. Thus, the W-Lys-X species are highly versatile molecules, which one can subject to the great diversity of peptide and protein conjugation and cross-linking reactions as known in the art (e.g., G. T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, 1996) to fabricate a diversity of LPS species. For LPS applications in which a biotin group is not needed, the synthesis of the LPS species can be simplified accordingly. Biotin analogs such as haptens (e.g., digoxin, digoxigenin, peptides) that are recognized by antibodies can be used instead of biotin, as can a common PNA sequence that every LPS species would contain in addition to its unique PNA sequence.

For solid phase synthesis of LPS species, the resin-attached precursor of W-Lys-X, in which the free end terminates in a primary amino group, can be reacted with N-succinimidyl-3-(2-pyridyldithio)propionate instead of succinic anhydride. The resulting pyridyldithiopropyl products can be array-spotted onto a thiol-substituted solid surface, such as thiol-substituted resin beads. The residual thiol groups on the surface can be blocked with iodoacetamide, and then ligands can be coupled through various linkages onto the lysine group of the immobilized PNAs. The resulting surface-attached LPS species can be released from the surface by dithiothreitol followed by blocking of the thiol group on these released species with iodoacetamide. Solid phase reactions can be convenient since residual reagents from the reactions can be removed by washing.

A collection of pre-assembled molecules, each containing a carboxyl group, a biotin, and a unique ligand can be attached to a resin-attached PNA precursor bearing a free amino group by activating the carboxyl group of the pre-assembled molecules with carbodiimide, or by forming a related active ester, for coupling onto the precursor PNA followed by release of the product from the resin. These pre-assembled molecules can be synthesized as follows. Nα-Boc-lysine (Sigma) is reacted with biotinamidohexanoic

acid N-hydroxysuccinimide ester (Sigma), the Boc group is removed under acidic conditions, and the product is reacted with ε-maleimidocaproic acid NHS ester (Sigma) followed by reaction with thiol-substituted ligands. These kinds of compounds can also be prepared by solid phase synthesis. Combinatorial ligands with carboxyl functional groups can be reacted directly with Nα-Boc-lysine, and then, after Boc removal, biotin can be attached.

Referring to Fig. 2A, a general way to discover complementary ligands and proteins according to the invention is illustrated. For clarity, a simple case is shown where just two LPS species, termed LPS_1 **22** and LPS_2 **24** are present. In this Figure, LPS is shown as a PNA molecule with a ligand, and biotin as a signal species, attached to the same end of the PNA, but other configurations can be used. For example, the ligand and biotin moieties can be attached to opposite ends of a PNA molecule, for example, by using the dual-labeling strategy reported elsewhere (Liu, X., Balasubramanian, S., Strategies for the synthesis of fluorescently labeled PNA, Tetrahedron Letters, 41, 6153-6156, 2000).

Referring again to Fig. 2A in step 1, LPS_1 **22** and LPS_2 **24**, prepared as described earlier, are exposed to a collection of proteins, where LPS_1 alone is able to form a complex. In step 2a, the reaction mixture is exposed to a separation step, e.g., ultrafiltration separation, where protein/LPS_1 is retained because of the large size of the protein, whereas LPS_2 **24**, since it is an unbound small molecule, passes through the membrane. After washing via ultrafiltration is carried out to thoroughly remove LPS_2, dissociating conditions are applied to the membrane-retained protein/LPS_1 complex so that further ultrafiltration, in step 2b, brings LPS_1 **22** through the membrane, leaving protein behind. There are two options for step 3, in which the positive LPS species (LPS_1 **22**) is detected. In option 3a, LPS_1 **22** hybridizes onto a sequence-complementary DNA (or PNA) arrayed spot on a solid surface. There is a complementary DNA (or PNA) spot on the spot-

arrayed surface for every LPS that is combined with the original protein sample. Subsequent addition of a streptavidin (SAv) signal species enables detection of the LPS_1 **22**. A streptavidin analog such as avidin or neutravidin may also be used. The specific location on the arrayed solid surface where the signal accumulates, and the known DNA (or PNA) oligomer at each site, initially establishes the identity of the LPS_1 species in terms of its PNA part. In turn, this information allows reverse tracing of the synthetic history of LPS_1, leading to the identification of the ligand part. Such reverse tracing of synthetic history to learn the structures of the compounds that test positive in a ligand assay is a standard procedure in many combinatorial synthesis techniques. More detail concerning the procedure of Fig. 2A is provided in Example 1.

Alternatively, in option 3b (comprising detailed steps of 3c-e shown in Fig. 2B) the LPS_1 **22** can be complexed via its biotin moiety onto a solid surface containing streptavidin or streptavidin-analog. In this case, the PNA code is detected via binding of a complementary double-stranded DNA probe, which, in turn, via a polymerase chain reaction (PCR) (steps 3c, d), yields complementary coded amplicons. In step 3c, the PCR reaction begins with the application of heat to dissociate the target DNA molecules to single strands, and then standard PCR amplification conditions in step 3d yield products called amplicons. In this example, one of the PCR primers is labeled with biotin, so one-half of each amplicon duplex product is biotin-labeled. These products are referred to as PCR Amplicons in Fig. 2B. The biotin-labeled strands of these amplicons can be specifically hybridized onto a complementary array of PNA (or DNA) spots on a solid surface followed by detection with signal-labeled streptavidin (step 3e). PNA spots are advantageous because they can provide intense binding for the biotin-labeled strand of the amplicons.

Referring again to Fig. 2A, the now-discovered ligands of the combinatorial synthesis library can be scaled up in step 4 for

preparation of affinity columns to isolate sufficient amounts of corresponding positive proteins for identification as by mass spectrometry. While detection via step 3b is more complex than detection by step 3a, the 3b option, via the PCR reaction, provides ultrasensitivity, which can be useful, for example, to study the proteome from a single biological cell, or from a relatively small number of such cells.

The method of the invention can also be practiced using techniques other than ultrafiltration to separate protein/ligand complexes from residual, unbound ligands. For example, chromatographic techniques such as size exclusion, ion exchange, immobilized metal, reversed-phase and hydrophobic interaction chromatography can be used in addition to ultrafiltration, as can electrophoretic techniques such as zone electrophoresis and isoelectric focusing, including capillary electrophoresis and capillary electrochromatography forms of these techniques. Isopycnic centrifugation can also be used by preparing LPS species that have a different buoyant density from that of proteins so that free and protein-complexed LPS species give rise to different bands upon centrifugation. For example, alkyl-substitution of LPS species can reduce their buoyant density while iodo-substitution, e.g., with diatrizoic acid, can increase their buoyant density. In general, proteins tend to have the same buoyant density aside from special classes such as lipoproteins.

PNA molecules are particularly attractive as the coding labels in the assay technique of the invention for several reasons. Most importantly, the coding ability of PNA molecules is high. For example, a 10-mer offers more than one million different codes. Furthermore, because these species are made up of noncharged, moderately polar substituents in a linear configuration, they can have good solubility in water and exhibit minimal nonspecific binding properties. DNA labels would make poor substitutes for PNAs since the intense charge of DNA labels would tend to cause much nonspecific binding in a multiplexed

ligand/protein assay. The peptide backbone of the PNA species provides high chemical and physical stability. These molecules are easy to synthesize and decorate with substituents in various geometries to create a variety of LPS species. A large number of LPS species, for example, more than 100, or even more than 10000, can be employed together for the simultaneous detection of an equivalent number of complementary proteins.

Some of the proteins of proteomes are present in very small numbers in cells. The small numbers generally reflect their special importance in the cell. These proteins are particularly difficult to discover with prior technology due to the sensitivity challenge. The methods of the invention, particularly in the PCR mode, open up a new way to reach the detection of such special proteins.

Discovery of ligands for proteins is important not only for the identification of complementary proteins, but also for the testing of positive ligands as probes in cellular assays to discover interesting features of cellular structure and function. This general strategy has been referred to as chemical genetics and is of great interest as a new way both to discover drugs and to gain more understanding of cellular biochemistry (Tan, D. S., Foley, M. A., Shair, M. D., Schreiber, S. L., Stereoselective Synthesis of over Two Million Compounds Having Structural Features Both Reminiscent of Natural Products and Compatible with Miniaturized Cell-Based Assays, J. Am. Chem. Soc., 120, 8565-8566, 1998).

With the discovery of protein-ligand binding partners by the methods of the invention comes the opportunity to create ligand-based chips for measuring proteins in which many spots of ligands with known specificity are set up for capture of their complementary proteins. This includes the option of sandwiching a given protein between two ligands, one of which is surface attached, and the second of which bears a signal group for detection purposes. Alternatively, the other sandwich-binding

partner, instead of a second ligand, can be a labeled antibody or antibody fragment. The small size of the ligands can help to minimize nonspecific binding. In the non-sandwich case, bound proteins are detected, e.g., by staining with dyes, or by covalent reaction with biotin NHS ester followed by streptavidin signal labeling. In some cases the protein samples are labeled before exposure to a protein chip. The bound proteins in the non-sandwich case can also be detected by mass spectrometry, e.g., by adding matrix and carrying out MALDI-TOF-MS analysis. Alternatively, adsorbed protein spots can be digested with trypsin to peptides before analysis by MS.

In a second embodiment according to the invention, known binding proteins such as antibodies, antibody fragments, phage display products and receptors are used in competitive and noncompetitive ligand assays to quantify analytes such as drugs, hormones, metabolites, toxins and other proteins. Multiplexing is achieved by using PNAs as the labels in these assays, attached either to ligands that compete with the binding proteins, or attached to the binding proteins, depending on the assay principle. As shown in Fig. 3, such assays can be divided into at least three types (I, II, and III). Also shown is a type IV assay in which a protein analyte is detected via sandwich binding between a ligand and a binding protein or between two ligands.

In a type I assay according to Fig. 3, one or more analytes are quantified together in a sample by adding, for each analyte, a distinctive, complementary protein and an LPS species consisting of a distinctive peptide nucleic acid part covalently attached to a distinctive ligand part L and a signal part S. Each target analyte and corresponding LPS compete for binding to their complementary protein. When a higher amount of a given analyte is present, the corresponding protein-bound form of LPS is decreased and the free form of LPS increases. Either form of the LPS can be measured to quantify the amount of the corresponding analyte. Usually the free and bound forms of LPS are separated before this

measurement. Detection takes place via hybridization of the free or bound form of LPS onto an array of PNA or DNA spots on a solid surface, based on the PNA and signal groups of the LPS tag. When protein bound LPS is detected, it may be preferred to dissociate the LPS first from the protein so that the protein cannot block the LPS from reaching its complementary PNA or DNA strand on the solid surface, or interfere with the detection of the signal group on the LPS. One can also take advantage of the resistance of the protein-bound LPS to be detected, since free LPS can then be measured in the presence of protein-bound LPS, so that the separation step in the method can be omitted.

In a type II assay, one or more analytes are quantified together in a sample by adding, for each analyte, a distinctive complementary protein, usually in excess relative to its target analyte. This protein previously was covalently labeled with a molecular species PS where P is a distinctive peptide nucleic acid and S is a signal group. The given protein binds its corresponding analyte, forming a soluble complex. A solid-phase distinctive ligand substance then is added for each analyte to bind residual protein that has not formed a complex, and at least the PS part of each complex is detected by a hybridization reaction via binding to a complementary PNA or DNA spot on a solid surface. Before this detection step, it is preferred to cleave the PNA-signal from the binding protein (release PS from Ab) to enhance the detection of PS via hybridization. This step can be implemented by incorporating a trypsin-cleavable group into the PNA (Weiler, J., Gausepohl, H., Hauser, N., Jensen O. N., Hoheisel, J. D., Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays, Nucleic Acids Research, 25, 2792-2799, 1997).

In a type III assay, a sample containing one or more analytes of interest is combined with one or more analyte-complementary protein pairs, one pair for each analyte, wherein one member of each protein pair is PNA-labeled; at least one of

the analytes becomes sandwiched between an analyte complementary protein pair, and at least one protein pair/analyte sandwich is detected via hybridization of its PNA part to an array of complementary PNA (or DNA) spots on a solid surface. Analyte is necessary for the sandwich complex to form, and, in turn, this complex must exist in order for the signal group of this complex to localize on the surface at the DNA or PNA spot designated for a given analyte based on the distinctive PNA that is part of the complex for that analyte.

The type IV assay is a sandwich assay just like the type III assay, except that one or both of the pairs of binding partners for the analyte is a ligand. This restricts the type IV assay to protein analytes, since high affinity binding is necessary in a sandwich complex for quantifying analytes reliably, and ligand/ligand interactions usually would be too weak for quantification. In the type IV assay, then, each protein analyte is complexed between a complementary signal-labeled ligand and a complementary PNA-labeled ligand or between a complementary protein and a complementary ligand, where the protein and the ligand are labeled with either signal or a PNA. As above, the sandwich complexes are detected via hybridization to a complementary PNA or DNA arrayed spot on a solid surface, based on the signal group of each complex.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE 1

Discovery of complementary ligand-protein binding pairs

A library of LPS species is obtained as shown in Fig. 1. A proteome is obtained as described (Sreekumar, A., Nyati, M. K.,

Varambally, S., Barrett, T. R., Ghosh, D., Lawrence, T. S., Chinnaiyan, A. M., Profiling of Cancer Cells Using Protein Microarrays: Discovery of Novel Radiation-regulated Proteins, Cancer Res., 61, 7585-7593, 2001) or by related technique. Fractions of this proteome can be obtained by chromatography such as size exclusion or ion exchange chromatography, or by isoelectric focusing. The proteome of a single cell can be obtained by pulling a cell of interest into a micropipet under a light microscope, and then lysing the cell hypotonically or with detergent. The LPS library and proteome (or proteome fraction) are combined and incubated in buffer at neutral pH, and subjected to ultrafiltration (e.g., using an ultrafiltration membrane from Amicon or Millipore) by means of centrifugation, pressure or vacuum. The retained, complementary protein/LPS pairs (containing positive LPS species) are subjected to washing steps with buffer to thoroughly remove noncomplexed LPS species. Dissociation of the protein/LPS complexes is accomplished by adding a different solution such as high pH buffer, low pH buffer, aqueous-organic solvent mixture, aqueous detergent, aqueous chaotropes, aqueous urea or a combination of these. The temperature also can be raised. Ultrafiltration then yields the dissociated, positive LPS species in the ultrafiltrate. For detection option 3a of Fig. 2A, this solution is then exposed to a solid surface containing an array of PNA or DNA spots such that there is a complementary coded spot for each species in the original LPS library. Nonbound LPS species are washed away with buffer or one of the above types of dissociation solutions. Spots that contain complexed LPS species can be detected by adding, e.g., streptavidin or streptavidin analog labeled with a signal group such as an enzyme (e.g., alkaline phosphatase), fluorophore (e.g., phycoerythrin, Cy5), or radioisotope (e.g., ^{125}I, ^{32}P). Once the positive spots are identified, then the identity of the PNA part of the positive LPS species is known, which in turn, enables, by retracing the synthetic history of the positive LPS species, the identity of the

ligand to be determined. Large quantities of the ligand then are synthesized and used to prepare an affinity column to isolate the complementary proteins in sufficient quantity for their identification by mass spectrometry. The positive LPS species obtained after ultrafiltration also can be identified in some cases by mass spectrometry. If the LPS species contains a reversible linkage (e.g., disulfide) between the ligand and the PNA, the PNA part can be dissociated and identified alone by mass spectrometry.

EXAMPLE 2

Multiplexed Competitive Ligand Assays with LPS

Antibodies are raised or obtained commercially against analytes of interest, or receptors for the analytes are obtained. Each of the analytes of interest is conjugated with a unique PNA. Alternatively, a ligand that cross-reacts with the antibody or receptor for a given analyte is conjugated with a PNA. Each LPS species is referred to as a tracer, and the antibody or receptor as the binder. A standard curve is set up for each analyte by incubating increasing amounts of the analyte as solutions in a series of containers with a fixed amount of complementary tracer and binder, wherein the amounts of the tracer, binder and analyte are similar (e.g., within a factor of 50). After equilibrium is established, in which the tracer and analyte compete for complexation with the binder, free (noncomplexed) and bound tracer are separated as by ultrafiltration, electrophoresis, or precipitation of bound tracer with a second antibody or with polyethylene glycol. Free tracer is then quantified by hybridization to a PNA or DNA arrayed spot on a solid surface, followed by detection with signal-labeled streptavidin. The multiple tracers and corresponding multiple binders can then all be combined, and a multiplexed standard curve is obtained similarly via incubation of the cocktail of tracers and binders with the corresponding standard analytes of interest throughout a

range of concentrations, to set up a corresponding collection of standard curves, one for each analyte. The multiplexed ligand assay is then ready for the determination of the concentrations of a mixture of analytes, by using the standard curves to convert the amounts of the free tracers into the amounts of the unknown analytes. In the event that the bound form of the tracers prevents the PNAs of such tracers from hybridizing to a complementary PNA or DNA arrayed spot on a solid surface, then this can be used to advantage since it means that the separation step for the free and bound tracers can be omitted prior to measuring the free tracers on a PNA or DNA array.

EXAMPLE 3

Multiplexed Ligand Assays with PNA-Signal-Labeled Protein Binders and Solid Phase Ligand

A library of solid-phase ligands (e.g., ligands on beads) is prepared as by combinatorial synthesis, or the library members of this synthesis are released from the solid surface and re-attached to a set of beads well suited for ligand assays such as magnetic particles form DYNAL, yielding so-called "tracer particles." Using conventional bioconjugation reagents (e.g., G. T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, 1996), the technique for incorporating a trysin-cleavage site into a PNA (Weiler, J., Gausepohl, H., Hauser, N., Jensen O. N., Hoheisel, J. D., Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays, Nucleic Acids Research, 25, 2792-2799, 1997), and the type of methodology shown in Fig. 1, PNAs are prepared having the composition maleimide-trypsin cleavage site-PNA-biotin, termed "MTPB," and are conjugated, one-by-one, to a set of ligand/analyte binding proteins (such as antibodies, antibody fragments or receptors) to form PS-labeled products termed "binders," while keeping track of which MTBA is attached to which binder. An assay is set up by combining binders and analytes, and then later the tracer particles are added to remove

uncomplexed binders, e.g., by magnetic separation, filtration or centrifugation. The residual, dissolved analyte/binder complexes are measured by digestion with trypsin to release the PS moieties, which in turn are reacted onto an array of PNA or DNA spots on a surface followed by detection with a streptavidin-signal reagent.

EXAMPLE 4

Multiplexed Sandwich Ligand Assays with PNA-Labeled Protein Binders

For each sufficiently-large analyte of interest in a collection of analytes, complementary protein, protein pairs are set up (e.g., by preparing or buying antibodies, antibody fragments, receptors) that form a sandwich complex consisting of protein/analyte/protein. A signal group such as biotin, an enzyme or a fluorophore is attached in a noninterfering way to one complementary protein, one for analyte pair, and a PNA is attached similarly to the other protein member. A collection of these analytes then can be detected simultaneously by incubating with the complementary protein pairs, followed by exposure to an array of complementary PNA or DNA spots on a solid surface, washing and detection of the signal group. A trypsin-releasable PNA can be used in this assay, so that PNA molecules released from the sandwich complexes are detected.

EXAMPLE 5

Multiplexed Sandwich Ligand Assays of Protein Analytes with PNA- and Signal-Labeled Ligands, or with PNA-Labeled Ligands and Signal-Labeled Proteins

This assay is conducted in the same way as Example 4, except that the analytes are necessarily proteins and the pair of sandwich reagents for each protein are either a PNA-labeled ligand and signal-labeled binding protein, or a PNA-labeled ligand and a signal-labeled ligand, where the two sandwich reagents for each protein analyte do not compete in their binding to the analyte. An assay can comprise a mixture of the two kinds of sandwich

pairs, where each protein analyte is targeted by one pair or another.

USE

The PNA-based ligand/protein binding assays of the invention have advantages both in their discovery and quantitation modes. In the discovery mode, complementary ligands and proteins are mutually discovered at the same time. With existing technology, individual proteins must be isolated and labeled before they are ready for screening against natural products or combinatorial synthesis compounds for ligand discovery. The large numbers of proteins, all of which are important, in all forms of life, make this approach to global ligand/protein discovery very onerous, to say the least. Further, the binding properties and stability of a protein may be degraded when it is labeled. Some proteins inherently are not very stable, and are especially difficult to label and purify. Also, relatively large quantities of a target protein may be needed with current technology.

Instead, the methods of the invention permit the replacement of this complex process by a scheme in which many proteins, even an entire proteome, can be combined directly, without any need for prior protein purification or labeling, in the solution phase with a candidate ligand library, based on the convenient preparation of ligand-PNA-signal (LPS) conjugates as a tag on the ligand. Positive LPS species can be isolated by a step as simple as ultrafiltration. Ultrafiltration membranes are widely employed to remove small, unbound molecules from proteins and protein complexes. Ultimately, detection is based on array detection on PNA or DNA spots, another technology that is also well established. Thus, the invention pulls together convenient, well-established chemical and physical tools and techniques in a new way to make a major advance in technology for discovering ligand/protein binding pairs. Every protein is significant, and having a ligand for every protein can not only speed up drug discovery, but increase our ability to study and control proteins

including their quantitation. For example, towards a goal of understanding the cellular function of proteins, it has been stated that, "for the chemical genetic approach to have its maximal impact, efficient methods of ligand discovery will be required to provide, in the limit, a small molecule partner for every gene product." (Tan, D. S., Foley, M. A., Shari, M. D., Schreiber, S. L., Stereoselective Synthesis of over Two Million Compounds Having Structural Features Both Reminiscent of Natural Products and Compatible with Miniaturized Cell-Based Assays, J. Am. Chem. Soc., 120, 8565-8566, 1998).

Another advantage of the invention is that it provides several options for quantifying proteins. These options exist in both the first and second embodiments. It is well-known that the properties of proteins cover a wide range in size, shape, charge, polarity and stability. This makes it difficult for any single assay technique to provide optimum conditions including ligands and binding reagents for quantitation of all of the proteins. Also, there may be trade-off in speed, cost, accuracy and degree of multiplexing among different assays. By offering a variety of assay principles and conditions, the methods of the invention help to cope with the diversity of proteins and analytical requirements in different studies. Which assay option is best for a given situation depends on the above considerations and is sorted out empirically and by experience.

The methods of the invention help to cope with the problem of protein diversity in a second way. This is due to the use of solution-phase conditions for the ligand/protein binding reactions. It is difficult to create a solid surface, especially where binding proteins themselves are on the surface, that is compatible (inert) towards all nontarget proteins. Indeed, this is a major problem that faces the development of protein chips (Service, R. F., Searching for Recipes for Protein Chips, Science, 294, 2080-2082, 2001). In the methods of the invention, an array of hybridization reactions on a solid surface can be used instead

of an array of protein binding reactions on a solid surface. Nonspecific binding is much less of a problem with hybridization than with protein-binding solid surfaces, especially when the hybridization reagents involve PNAs, because of the small size, lack of charge, moderate polarity and high affinity hybridization reactions. It is novel to detect proteins via PNA or DNA chips, and very practical because the technology for using these chips is so well established.

While the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the foregoing description. Accordingly, it is intended that the invention shall be directed to all such alternatives, modifications and variations as they fall within the spirit and broad scope of the appended claims.

CLAIMS

What is claimed is:

1. A method for detecting protein - ligand binding, said method comprising the steps of:

providing one or more ligands, wherein said ligands are each covalently attached to a tag, said tag comprising a peptide nucleic acid species, and wherein, further, each said peptide nucleic acid species uniquely identifies said ligand;

incubating said tagged ligands with a sample comprising one or more proteins whereby one or more protein/tagged ligand complexes can form; and

identifying said peptide nucleic acid species tag portion of said complexed tagged ligand.

2. The method of claim 1, wherein said tag further comprises a signal species.

3. The method of claim 2, wherein said signal species is selected from the group consisting of biotin, a fluorescent species, a radioactive species and a generic peptide nucleic acid species.

4. The method of claim 2, wherein, in said tag, said signal is attached to a different portion of said peptide nucleic acid species compared to the portion of said peptide nucleic acid species attached to said ligand.

5. The method of claim 1, further comprising, prior to said identifying step, the step of separating any protein/tagged ligand complexes formed in said incubating step from non-complexed said tagged ligand.

6. The method of claim 5, wherein said separating step is ultrafiltration or centrifugation.

7. The method of claim 1, wherein, in said incubating step, said sample is from a biological cell.

8. The method of claim 1, wherein, in said incubating step, said sample comprises unlabeled said one or more proteins.

9. The method of claim 1, wherein, in said providing step, said one or more ligands are from a combinatorial library.

10. The method of claim 1, wherein said identifying step comprises binding said peptide nucleic acid species to a solid surface and detecting said binding.

11. The method of claim 1, wherein said identifying step comprises binding said tag to a solid surface and detecting said binding by hybridization of said peptide nucleic acid species.

12. The method of claim 1, wherein said one or more tagged ligands comprise more than 100 different species.

13. The method of claim 1, wherein said sample comprises more than 100 different proteins.

14. The method of claim 5, further comprising, prior to said identifying step, the step of dissociating said protein/tagged ligand complexes into protein and tagged ligand portions.

15. A method for detecting protein - ligand binding, said method comprising the steps of:

providing one or more ligands, wherein said ligands are each covalently attached to a tag, said tag comprising a peptide

nucleic acid species and biotin, and wherein, further, each said peptide nucleic acid species uniquely identifies said ligand;

incubating said tagged ligands with a sample comprising one or more proteins whereby one or more protein/tagged ligand complexes can form; and

identifying said peptide nucleic acid species tag portion of said complexed tagged ligand, said identifying step comprising binding said biotin portion of each tag of a complexed said tagged ligand to avidin or an avidin analog on a solid surface and detecting said peptide nucleic acid portion of each tag by hybridization followed by PCR amplification.

16. A method for assaying an analyte, said method comprising the steps of:

providing one or more ligands, wherein said ligands are each covalently attached to a tag, said tag comprising a peptide nucleic acid species, and wherein, further, each said peptide nucleic acid species uniquely identifies said ligand;

providing one or more complementary ligand-binding proteins, one for each said one or more ligand;

incubating said one or more tagged ligands and said one or more complementary ligand-binding proteins with a sample that contains one or more cross-reacting analytes of interest for said one or more proteins whereby one or more of said analytes competitively reduces the fraction of tagged ligand bound by a complementary ligand-binding protein for said analyte and said tagged ligand; and

quantifying either said peptide nucleic acid species tagged to ligand bound by said complementary ligand-binding protein or said peptide nucleic acid species tagged to ligand not bound by said complementary ligand-binding protein, said quantifying step comprising binding said peptide nucleic acid portion of each tag of either said bound or not bound fraction by hybridization to a complementary species on a solid surface.

17. The method of claim 16, further comprising, prior to said quantifying step, the step of separating any protein-bound tagged ligand formed in said incubating step from unbound said tagged ligand.

18. A method for assaying an analyte, said method comprising the steps of:

providing a sample comprising one or more analytes of interest;

incubating said sample with one or more analyte-binding proteins, one for each said analyte, wherein said analyte-binding proteins are each covalently attached to a tag, said tag comprising a peptide nucleic acid species, and wherein, further, each said peptide nucleic acid species uniquely identifies said analyte-binding protein, whereby one or more of said analytes are bound by complementary said analyte-binding proteins; and

quantifying said peptide nucleic acid species tagged to analyte-binding protein bound to said analyte of interest, said quantifying step comprising binding said peptide nucleic acid portion of each tag by hybridization to a complementary species on a solid surface.

19. The method of claim 18, further comprising, prior to said quantifying step, the step of separating any analyte-bound tagged protein formed in said incubating step from unbound said tagged protein.

20. A method for carrying out a sandwich assay for an analyte, said method comprising the steps of:

providing a sample comprising one or more analytes of interest;

incubating said sample with one or more complementary protein pairs, one for each said analyte, wherein one member of

each said protein pair is covalently attached to a tag, said tag comprising a peptide nucleic acid species, and wherein, further, each said peptide nucleic acid species uniquely identifies said member of said protein pair, whereby at least one of said analytes is sandwiched between the members of said complementary protein pair; and

quantifying said peptide nucleic acid species tagged to said protein pair member of said protein pair-analyte sandwich, said quantifying step comprising binding said peptide nucleic acid portion of each tag by hybridization to a complementary species on a solid surface.

21. The method of claim 20, wherein, further, in said incubating step, said other member of each said protein pair is covalently attached to a signal.

22. A method for carrying out a sandwich assay for a protein analyte, said method comprising the steps of:

providing a sample comprising one or more protein analytes of interest;

incubating said sample with one or more complementary ligand pairs or complementary ligand-protein pairs, one for each said analyte, wherein one member of each said pair is covalently attached to a tag, said tag comprising a peptide nucleic acid species, and wherein, further, each said peptide nucleic acid species uniquely identifies said member of said pair, whereby at least one of said protein analytes is sandwiched between the members of said complementary pair; and

quantifying said peptide nucleic acid species tagged to said pair member of said ligand-ligand or ligand-protein pair-analyte sandwich, said quantifying step comprising binding said peptide nucleic acid portion of each tag by hybridization to a complementary species on a solid surface.

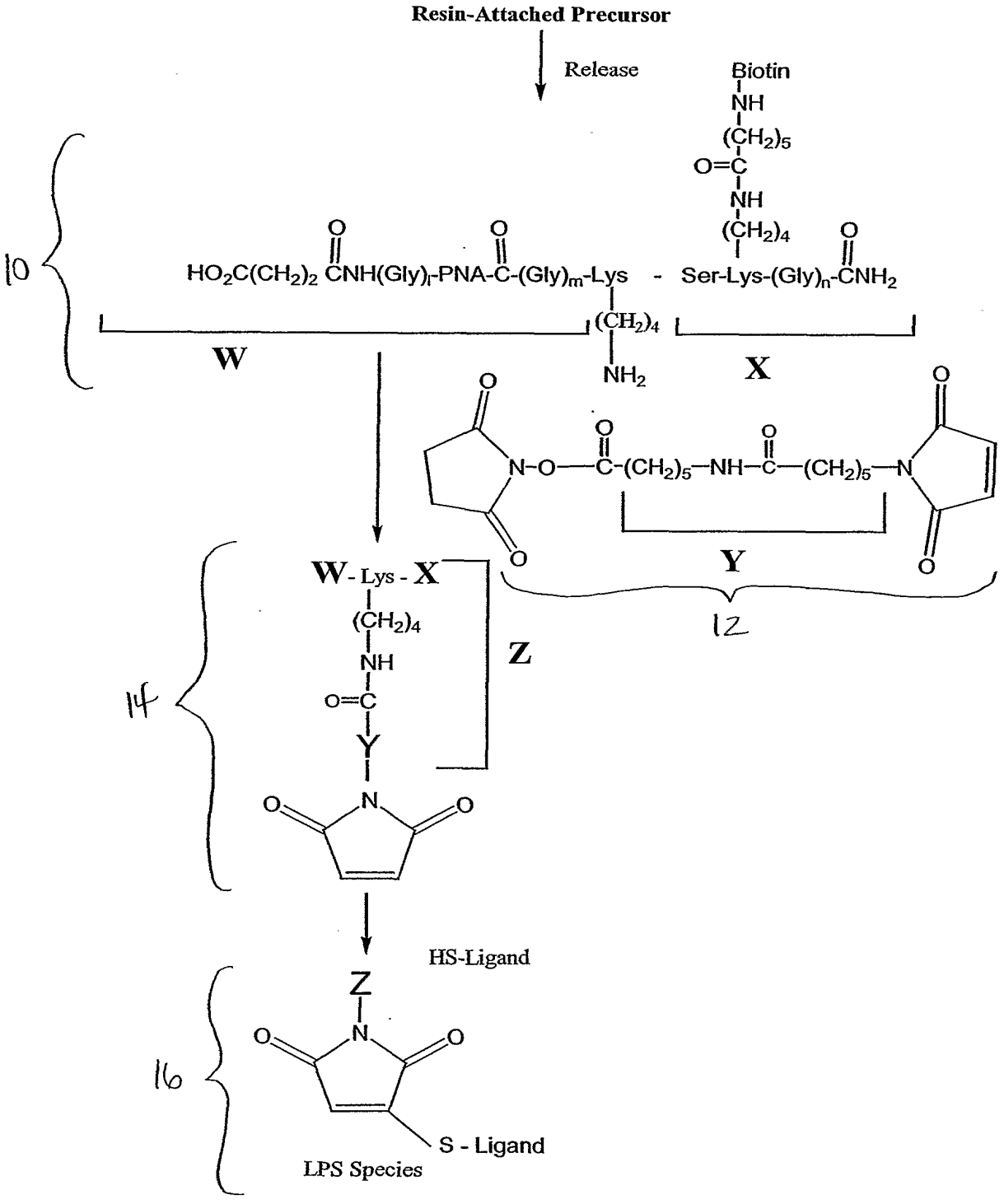
23. The method of claim 22, wherein, further, in said incubating step, said other member of each said pair is covalently attached to a signal.

24. A tagged ligand for proteome analysis comprising a ligand prepared originally by combinatorial synthesis, wherein said ligand is covalently attached to a tag, said tag comprising a peptide nucleic acid species and a signal species, and wherein, further, each said peptide nucleic acid species uniquely identifies said ligand.

25. The tagged ligand of claim 24, wherein said signal species is selected from the group consisting of biotin, a fluorescent species, a radioactive species and a generic protein nucleic acid species.

26. The tagged ligand of claim 24, wherein said tagged ligand is used to identify ligands or proteins of interest.

27. The tagged ligand of claim 24, wherein said tagged ligand is used to quantify ligands or proteins of interest.


Resin-Attached Precursor
Release
Biotin
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(CH2)5
O=C
NH
(CH2)4
HO2C(CH2)2 CNH(Gly)l-PNA-C-(Gly)m-Lys - Ser-Lys-(Gly)n-CNH2
(CH2)4
NH2
10
W
X
N-O-C-(CH2)5-NH-C-(CH2)5-N
Y
12
W- Lys - X
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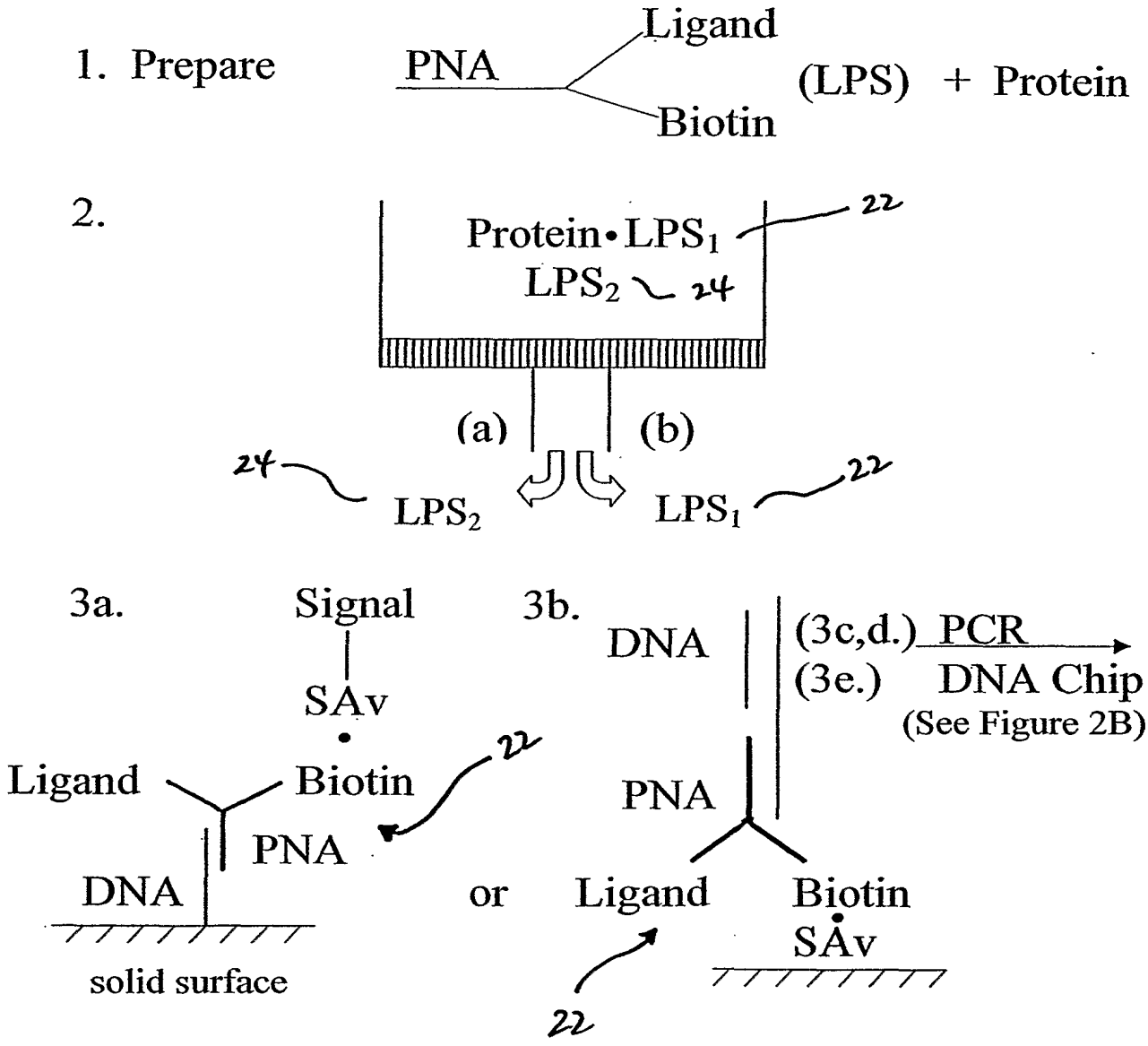
NH
O=C
Y
Z
14
HS-Ligand
Z
16
S - Ligand
LPS Species

Fig. 1



4. Isolate protein on L affinity column for identification.

Fig. 2A

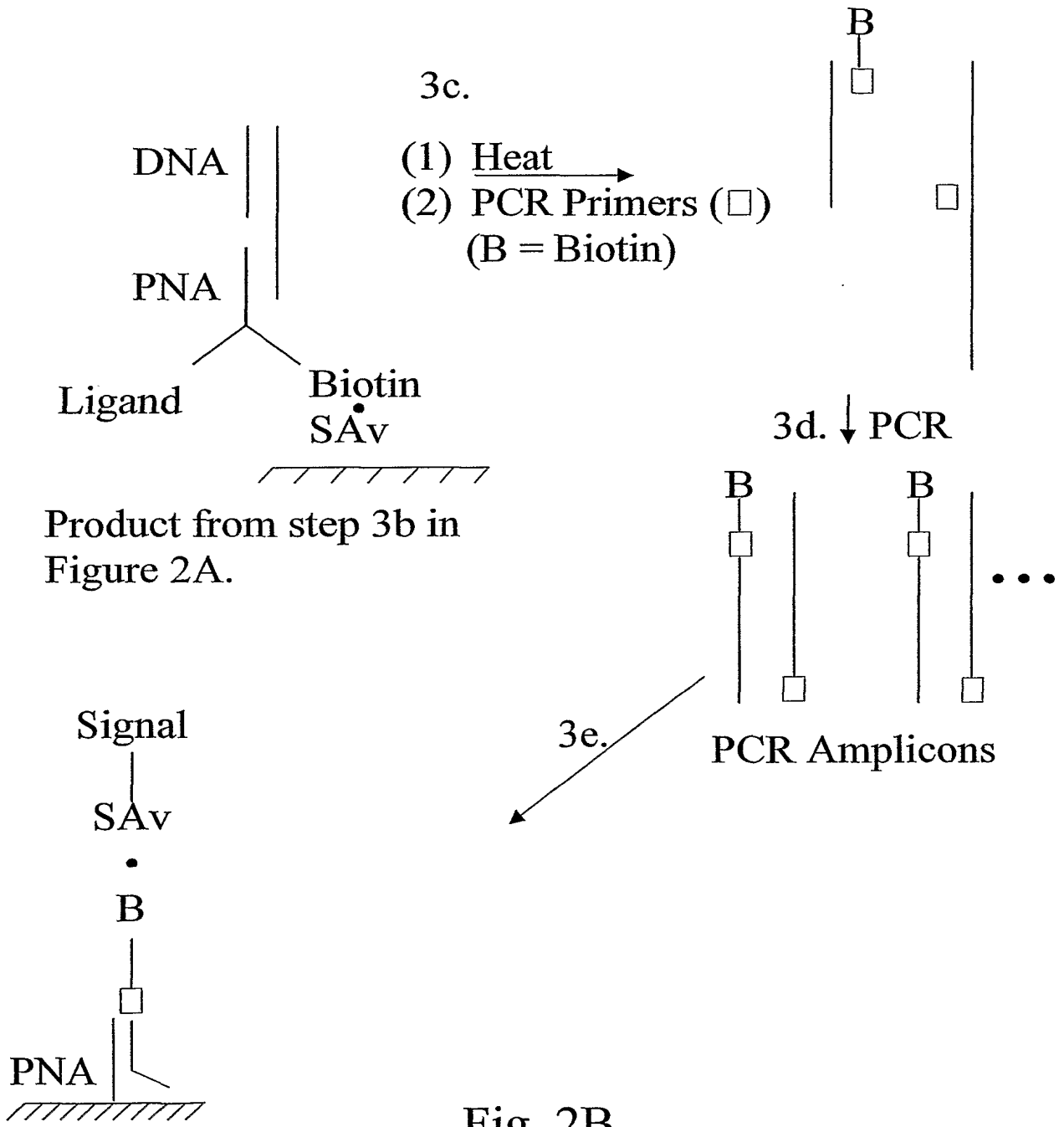

3c.
DNA
PNA
Ligand
Biotin
SAv
(1) Heat
(2) PCR Primers (□)
(B = Biotin)
B
3d. PCR
B
B
Product from step 3b in Figure 2A.
3e.
PCR Amplicons
Signal
SAv
B
PNA

Fig. 2B

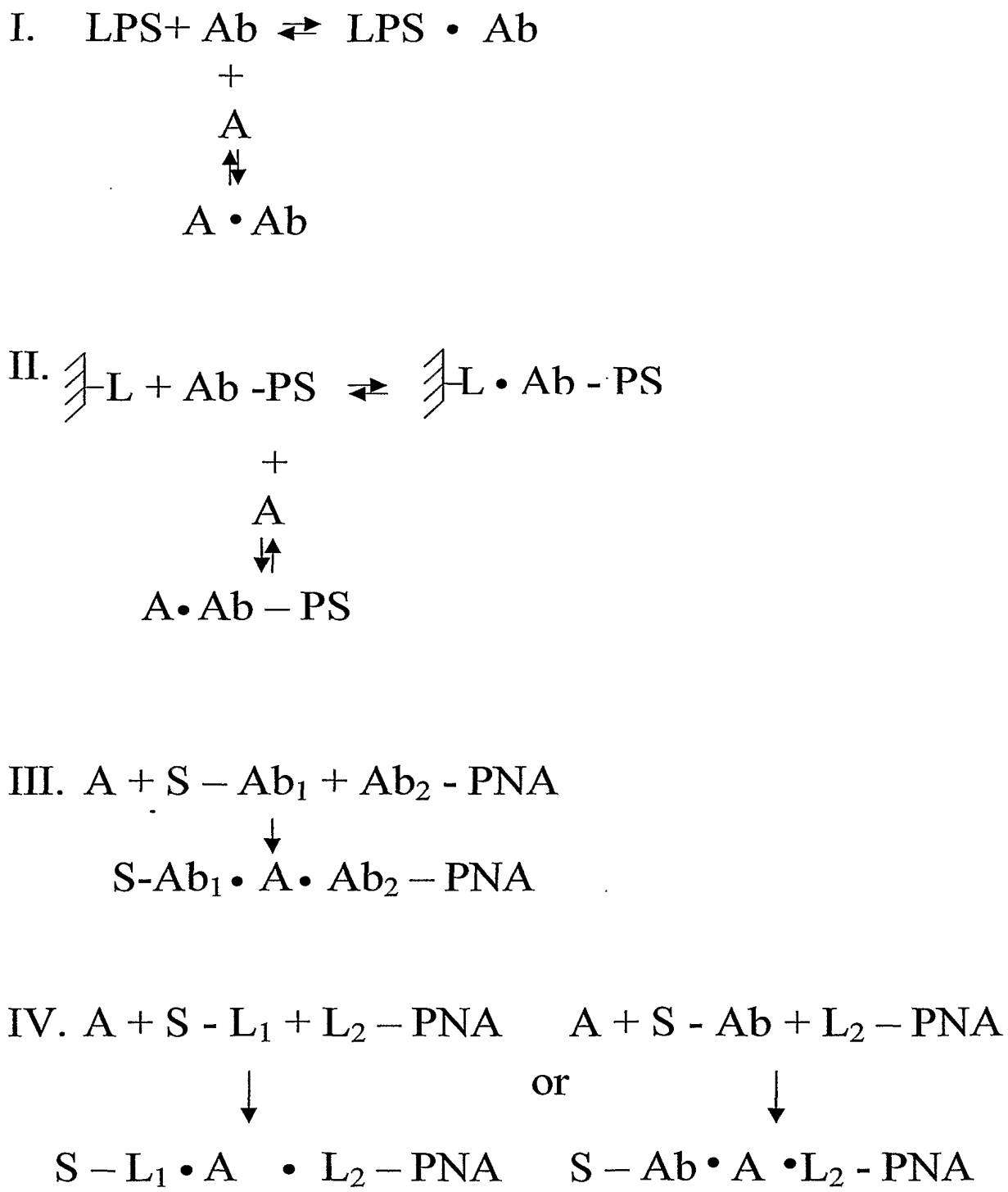

I. LPS+ Ab ⇄ LPS • Ab
+
A
A • Ab
II. L + Ab -PS ⇄ L • Ab - PS
+
A
A• Ab – PS
III. A + S – Ab1 + Ab2 - PNA
S-Ab1 • A • Ab2 – PNA
IV. A + S - L1 + L2 – PNA
A + S - Ab + L2 – PNA
or
S – L1 • A • L2 – PNA
S – Ab • A •L2 - PNA

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/11411

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/68
US CL : 435/6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,165,720 A (FELGNER ET AL) 26 December 2000 (26.12.2000), whole document.	1-27
A	US 6,060,242 A (NIE ET AL) 9 May 2000 (09.05.2000), whole document.	1-27
A	US 6,045,995 A (CUMMINGS ET AL) 4 April 2000 (04.04.2000), whole document.	1-27
A	EP 0 781 853 A2 (ELI LILLY AND COMPANY) 02 September 1997 (02.09.1997), whole document.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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25 June 2002 (25.06.2002)

Date of mailing of the international search report
09 AUG 2002

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